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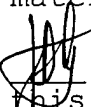designated by other documentation.

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Highly pure, native preparations of recombinant gp120 have been obtained from the T-tropic HIV-1$_{LAI}$ and the macrophage-tropic HIV-1$_{JR-FL}$ isolates. Conjugates of these HIV-1 gp120 preparations were prepared with tetanus toxoid (TT) and keyhole limpet hemocyanin (KHL) with the hypothesis that very immunogenic carrier proteins will increase the immunogenicity of gp120. Crosslinking procedures were selected that did not affect the ability of HIV-1 gp120 to bind to CD4. However, immunization studies in mice showed that anti-gp120 antibody levels were lower in animals immunized with conjugates compared with animals receiving HIV-1 gp120 alone. Moreover, repeated immunizations with either KHL-gp120 conjugates or gp120 alone failed to induce neutralizing antibodies to a HIV-1 clade B isolate. A new strategy has been designed to improve the immunogenicity of these HIV-1gp120 preparations. It is based on the capacity of DNA immunization to induce T cell-mediated helper function linked to booster immunizations with unconjugated gp120 preparations.

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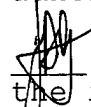
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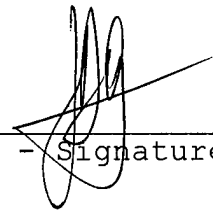
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INTRODUCTION

This annual report summarizes the work carried out during the first two years of funding, through 05-31-97. I was named the P.I. for this Grant on June 25th 1997; therefore, the work presented in this report was carried out under the responsibility of Dr. Tran Chanh, the previous P.I. I have prepared this report based on information provided to me by all scientists involved in the project. The co-P.I.s of this Grant, Dr. Kennedy (University of Oklahoma) and Dr. Maddon (Progenics Pharmaceuticals), and I have evaluated the data that have been generated during these first two years. Based on the scientific evidence of the results, we propose a series of experiments that are not exactly the same that were outlined in the original proposal. We believe that these experiments also reflect the current knowledge on HIV vaccine development and immunology that has emerged during the last three years.

RESULTS

Specific Aim #1: *Produce and characterize 100 mg of HIV-1 gp120 derived from LAI and JR-FL strains for use in conjugation and immunization experiments.*

Chinese Hamster Ovary (CHO) cell lines were produced that express high levels of the laboratory-adapted T-cell tropic HIV-1$_{LAI}$ or the primary macrophage tropic HIV-1$_{JR-FL}$ gp120 proteins. Supernatant of these cell lines was used for purification of secreted gp120. First, supernatants were passed through *Galanthus nivalis* (GNA) columns, which binds glycans containing terminal mannose residues, and then glycoproteins were eluted with methyl-α-D-mannopyranoside. The gp120 preparations were loaded onto Q High Performance anion exchange columns, eluted with step-wise increases of sodium chloride concentrations, concentrated and loaded onto a Superdex 200 gel filtration column. The level of purity and integrity of the final product was confirmed by SDS-PAGE, and it was shown to be higher than 95% for both gp120 preparations. As it is deduced from the nucleotide sequence, HIV-1$_{LAI}$ gp120 contains

13 more amino acids and one more site for potential N-linked glycosilation than HIV-1$_{JR-FL}$, and these differences were reflected in their gel mobility.

The aggregation state of the purified gp120 proteins was determined by analytical gel filtration and it showed that the three-column purification process yielded proteins that were >95% monomeric. The conformational integrity of the purified recombinant HIV-1$_{LAI}$ and HIV-1$_{JR-FL}$ gp120 proteins was assessed by binding to soluble CD4 and CD4-IgG2 in an ELISA format. Both gp120 preparations bound to CD4-based proteins with nanomolar affinity, suggesting that they were conformationally intact. Furthermore, flow cytometry analysis showed that these gp120 preparations were able to bind to CHO cells that expressed CD4 on their surface. These results indicated that the recombinant HIV-1$_{LAI}$ and HIV-1$_{JR-FL}$ gp120 preparations were in their native conformation with intact CD4-binding sites.

Prior to conjugation, the purified gp120 preparations were tested for immunogenicity. Guinea pigs were immunized with five subcutaneous and intraperitoneal inoculations of 100μg of either gp120 strain in alum or Ribi Detox adjuvants. The humoral immune response was determined by ELISA titers against the same protein preparation. Similar results were observed using HIV-1$_{LAI}$ and HIV-1$_{JR-FL}$ gp120 proteins; although the Ribi adjuvants elicited higher titers after the first inoculations, the maximum titers elicited by both adjuvants were similar.

Antibodies capable of preventing binding of gp120 to CD4, should neutralize a broader range of HIV-1 strains than antibodies directed to epitopes other than the VP3 loop. Sera from guinea pigs immunized with HIV-1$_{LAI}$ and HIV-1$_{JR-FL}$ gp120 proteins were tested for their capacity to compete with biotinylated gp120 for binding to soluble CD4 that had been adsorbed to ELISA plates. It was observed that all sera contained antibodies capable of blocking CD4 binding, but the highest titers we seen in animals immunized with Ribi adjuvant. These differences were presumably due to the denaturing effect of the alum adjuvant on the conformation of gp120.

In summary, milligram quantities of pure HIV-1$_{LAI}$ and HIV-1$_{JR-FL}$ gp120 were prepared. These preparations were shown to bind CD4, be monomeric, and immunogenic in guinea pigs.

Specific Aim #2: *Conjugate tetanus toxoid and diphtheria toxoid carriers to the gp120 molecules using a variety of established chemistries. Evaluate both the integrity of gp120 after conjugation and the immunogenicity of the HIV-1 subunit conjugates in mice.*

The hypothesis was that the incorporation of highly immunogenic antigen carrier into HIV-1 gp120, a heavily glycosilated envelope glycoprotein, will result in a more active immunologic recruitment of helper T lymphocytes and will significantly augment the anti HIV immune response. In order to test such hypothesis, tetanus toxoid (TT) was conjugated to purified gp120 using glutaraldehyde and mice were immunized with this conjugate in alum adjuvant. When compared to unconjugated gp120, mice immunized with TT-gp120 had lower anti-gp120 titers; these results were observed with both HIV-1$_{LAI}$ and HIV-1$_{JR-FL}$ gp120 preparations.

In summary, these poor immunogenicity results in mice, and the possibility that the presence of anti-TT antibodies in humans might cause immunosuppression to the gp120 component of TT-gp120 conjugates, made us switch to a different protein carrier. We selected the keyhole limpet hemocyanin (KHL) protein for conjugation studies. KHL is a very large protein (8.6 x 10^6 D) that has been shown to be very effective as carrier for peptide antigens and that has been used in many cancer vaccine candidates in humans (Helling et a., 1994; Longeneck et al, 1994).

Specific Aim #3: *Identify the optimal HIV-1 subunit conjugate vaccine and extensively characterize the immune response (and in vitro antiviral effects) in mice and baboons.*

Purified HIV-1$_{LAI}$ gp120 was conjugated to KLH by several chemical reactions that targeted different functional groups of gp120, such as carbohydrate moieties, primary amines, and carboxyl groups. The reactions included: 1) N-hydroxysuccinimidyl-2-3-dibromopropionate (SDBP) at 3 mg/ml; 2) SDBP at 6 mg/ml; 3) glutaraldehyde at 0.075% in a two-step procedure; 4) glutaraldehyde at 0.25% in a two-step procedure; 5) glutaraldehyde at 0.25% in a one-step procedure; 6) reductive

amination of glycan components of gp120 with sodium *m*-periodate; 7) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in a one- and two-step procedure; and 8) EDC in the presence or absence of sulfo-N-hydroxysuccimide. Conjugates were tested in a sandwich ELISA specific for gp120-KLH conjugates possessing a functional CD4-binding site. Appreciable CD4-binding activities were observed for conjugates prepared with 3 mg/ml of SDBP and with glutaraldehyde at 0.075% in a two-step procedure; SDS-PAGE analysis showed that for these conjugates 50-90% of gp120 was crosslinked to KHL. All other crosslinking procedures affected CD4-binding properties adversely. Since both the SDBP and the glutaraldehyde procedures yielded gp120-KHL conjugates of similar CD4-binding properties, we decided to further characterize glutaraldehyde conjugates. This decision was based on that we find the glutaraldehyde conjugation procedure more simple, reproducible and controllable.

To evaluate the immunogenicity of the HIV-1$_{LAI}$ and HIV-1$_{JR-FL}$ gp120 glycoconjugate preparations, we immunized groups of BALB/c mice and determined anti-gp20 endpoint titers by methods previously described (Wolf et. Al., 1992). Mice received multiple injections at monthly intervals of the gp120-KLH glycoconjugates (5 μg of gp120) in alum. The anti-gp120 titers were compared to groups of mice immunized with either the gp120 preparation (5 μg) in alum or in saline (Table 1). The gp120 administered in saline was weakly immunogenic. No anti-gp120 response was observed with either gp120 preparation until after the fifth injection. Three additional immunizations failed to significantly boost the anti-gp120 titers above that obtained following the fifth injection. The immunogenicity of the gp120 preparations was increased by employing alum as an adjuvant. IgG anti-gp120 titers were observed following the third (HIV-1$_{LAI}$ gp120) and second (HIV-1$_{JR-FL}$ gp120) immunization with the two different preparations. The anti-gp120 responses peaked following the fifth injection and with the HIV-1$_{LAI}$ gp120 isolate was boosted by additional vaccinations. The anti-gp120 response in the HIV-1$_{JR-FL}$ gp120 group remained stable following additional injections. These responses were compared to immunization with the glycoconjugates. Based on anti-gp120 titers, conjugation to KLH did not appear to enhance the immunogenicity of gp120 preparations. In fact, for both gp120

preparations, conjugation to KLH appeared to reduce immunogenicity based on anti-gp120 titers.

Table 1. Anti-HIV-1 JR-FL and LAI recombinant gp120 endpoint titers in sera from immunized mice.[a]

Group[b]	Injection Number	Anti-gp120 titer[c] (LAI)	Anti-gp120 titer (JR-FL)
gp120 soluble	1	<50	<50
	2	<50	<50
	3	<50	<50
	4	<50	<50
	5	230(50-800)	2,680(100-6,400)
	6	ND	ND
	7	ND	ND
	8	710(50-3,200)	3,220(100-6,400)
gp120 alum	1	<50	<50
	2	<50	4,240(400-6,400)
	3	1,010(50-3,200)	40,960(25,600-102,400)
	4	5,960(200-12,800)	37,120(6,400-102,400)
	5	8,960(3,200-12,800)	71,680(25,600-102,400)
	6	ND	ND
	7	ND	ND
	8	32,320(1,600-102,400)	30,720(25,600-102,400)
KLH/gp120 alum	1	<50	<50
	2	<50	1,320(200-3,200)
	3	710(50-3,200)	6,880(400-25,600)
	4	3,530(50-12,800)	32,320(1,600-102,400)
	5	2,090(50-3,200)	37,120(6,400-102,400)
	6	ND	ND
	7	ND	ND
	8	4,200(400-6,400)	21,760(6,400-25,600)

[a] Titers were determined indirectly by solid phase ELISA.
[b] Groups consisted of 5 female Balb/c mice, immunized and bled monthly.
[c] The values represent the reciprocal of the dilution of sera that was greater than 3 times the value of the individual pre-sera less background. The mean of each group is shown with the range of the individuals in parentheses.

Our criterion for evaluating prospective candidates in baboons was the ability of the anti-gp120 responses induced in mice to neutralize primary HIV-1 isolates. To determine whether gp120 immunized mice could neutralize primary HIV-1 isolates *in vitro*, we selected the primary clade B isolate, designated BZ 167. The BZ 167 was titrated on human PBMC target cells and growth curves were generated. Pooled serum

obtained from mice prior to injection, after the fifth injection and following the eight injection were diluted 1:50 and evaluate for their ability to neutralize BZ 167 activity. No neutralizing activity as assessed by a reduction of HIV-1 p24 activity was observed with any of the mouse sera (Table 2). Our positive control, a murine monoclonal anti-CD4 preparation (Attanasio et. Al., 1993) was capable of partially neutralizing 100 TCID$_{50}$ of BZ 167 on day 7 (60% inhibition) and day 10 (78% inhibition) of culture. These results indicated that the anti-gp120 antibody responses were not capable of neutralizing a primary clade B isolate *in vitro*.

Table 2. Neutralization of an HIV-1 clade B primary isolate (BZ 167) by murine anti-HIV-1$_{JR-FL}$ gp120 and gp120/KLH conjugate vaccine sera.[a]

	Day 7		Day 10	
Inhibitor	OD 490[b]	% Inhibition	OD 490	% Inhibition
none	1.182[c]	0	1.236	0
Anti-CD4	0.475	60	0.262	78
Pre-sera	1.054	11	1.348	0
SV40 Tag	1.084	8	1.232	0
KLH	1.062	10	1.212	2
Soluble gp120	1.130	4	1.360	0
gp120 Alum	1.169	11	1.219	1
gp120/KLH Alum	1.196	0	1.244	0

[a] Human PBMC blasts were infected with 100 TCID$_{50}$/ml HIV-1 BZ 167 in the presence or absence of various inhibitors.
[b] HIV-1 p24 levels were determined indirectly by solid phase ELISA.
[c] Values represent the mean of duplicate determinations.

In summary, KLH-gp120 conjugates were prepared by methods that still allowed for CD4-binding; however, immunogenicity studies in mice showed that these conjugates were less immunogenic than unconjugated gp120 preparation. Based on these results, it was deemed that additional neutralizing assays employing primary isolates from other clades and immunogenicity studies in baboons were not warranted.

CONCLUSIONS

Highly pure preparations of recombinant gp120 have been obtained from two different HIV-1 isolates. Conjugates of these HIV-1 gp120 preparations were prepared with tetanus toxoid (TT) and keyhole limpet hemocyanin (KHL) with the hypothesis that

very immunogenic carrier proteins will induce better antibody responses to gp120. Conjugation procedures were selected that did not affect the ability of HIV-1 gp120 to bind to CD4. However, immunization studies in mice showed that anti-gp120 antibody levels were lower in animals immunized with conjugates compared with animals receiving HIV-1 gp120 alone. Moreover, repeated immunizations with either KHL-gp120 conjugates or gp120 alone failed to induce neutralizing antibodies to a HIV-1 clade B isolate. Additional assays will be performed with the sera from mice immunized with different HIV-1 gp120 preparations. Mouse potency studies (Attanasio et. al., 1991) will be performed to determine whether conjugation results in a more potent immunogen based on the dose required to induce immune responsiveness. We will further characterize serologic reactivities by determining cross-reactivity to the different gp120 preparations by ELISA. We will also evaluate anti-gp120 fine specificity and *in vitro* neutralizing activity against the homologous isolates by methods previously described (Warren et. al., 1992). To determine if conjugation altered the T helper (Th) cell phenotype, we will also evaluate mouse IgG subclass response specific for gp120. The potential exists that conjugation of gp120 to a carrier protein may alter the presentation of the antigens and result in a change in the induction of Th cells. Should the ratio of IgG2a:IgG1 (Th1:Th2) anti-gp120 antibody response indicate a difference in the Th phenotype between mice immunized with gp120 alone versus the glycoconjugate, we will further examine T cells obtained from immune splenocytes and determine cytokine secretion by methods previously described (Bright et. al., 1995).

Another aspect of our future studies deals with the vaccination approach that we have followed. In general, subunit vaccines, like killed vaccines, do not provide endogenously synthesized proteins and, therefore, do not induce cytotoxic T cells. Some exceptions to this limitation in CTL priming have been found; subunit antigens that aggregate into virus-like particles and glycoproteins that are incorporated into lipid micelles have been demonstrated to induce antigen-specific CTLs. In contrary to killed and subunit vaccines, live-attenuated vaccines provide for long-lasting humoral and cell-mediated immunity. However, in some cases these vaccines may be immunosuppressive, cause clinical disease if not attenuated sufficiently, or be ineffective if attenuated too much. First described in 1990 (Wolff et al, 1990), the direct injection of genes encoding protective antigens into the host has the potential to solve many of the shortcomings of the new

generation vaccines. Genetic vaccines, or the use of antigen-encoding DNAs to vaccinate, represent a new approach to the development of subunit vaccines. In this case, the host cells take up the DNA, express the antigen-coding sequences and induce an immune response; the DNA persists for months and does not integrate into the cellular chromosomes. The coexpression of certain costimulatory cytokines can augment the immune response to plasmid DNA immunogens. For example, coexpression of GM-CSF and/or IL-12 restored CTLs response to a nonimmunogenic influenza virus nucleoprotein (NP) mutant (Iwasaki et al, 1997); coinoculation of plasmid DNAs encoding HIV-1 envelope and IL-12 resulted in enhanced HIV-1-specific cell-mediated immunity (Tsuji et al, 1997). DNA vaccine trials have been carried out in rhesus macaques in order to induce protection against SIV infection (Lu et al, 1996). Although the DNA vaccine raised both CTL and neutralizing antibody responses against SIV, it failed to provide sterilizing immunity against challenge with virulent SIVmac251; CTL responses were stable and remained detectable at the time of challenge, but the presence of neutralizing antibodies was transient and booster DNA immunizations failed to raise their titer. However, we have shown before that a combination of primary immunization with a live vector and booster inoculations with a subunit vaccine induces higher antibody titers than several inoculations with a live vector or a subunit vaccine alone (Giavedoni et al, 1993).

In summary, we will immunize two groups of baboons with plasmid DNA expressing HIV-1 gag, env and pol genes. To test for the immune enhancing effect of cytokines, one group will receive coinoculation of plasmid DNA encoding for GM-CSF and IL-12. In order to induce high titers of neutralizing antibodies, both groups will receive booster immunizations with a mixture of HIV-1$_{LAI}$ and HIV-1$_{JR-FL}$ gp120. Anti-HIV-1 neutralizing antibodies, cell-mediated immunity and cytokine profile will be determined for all animals.

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